

Structural and enzymatic characterization of a purified prohormone-processing enzyme: Secreted, soluble Kex2 protease

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ABSTRACT The prohormone-processing Kex2 protease of the budding yeast *Saccharomyces cerevisiae* can be converted from an intracellular membrane protein to a soluble, secreted, and active form by deletion of the transmembrane domain and C-terminal tail. One such molecule was purified to near homogeneity from the culture medium of an overexpressing yeast strain. Amino acid sequence analysis revealed that the N terminus of mature Kex2 protease is created by a potentially autoproteolytic cleavage at Lys¹⁰⁸-Arg¹⁰⁹, prior to the domain homologous to subtilisin, followed by trimming of Leu-Pro and Val-Pro dipeptides by the Ste13 dipeptidyl aminopeptidase. Kinetic parameters were examined using fluorogenic peptidyl-methylcoumarin amide substrates. Initial burst titration indicated that the preparation was entirely active. Measurements of dependence of activity on pH yielded a simple curve suggesting titration of a single ionizable group. Activity was half-maximal at pH 5.7 and nearly constant from pH 6.5 to 9.5. Discrimination between substrates was as great as 360-fold in K_m and 130-fold in k_{cat} . Substrates with a Lys-Arg dipeptide preceding the cleaved bond were preferred, having k_{cat}/K_m values up to $1.1 \times 10^7 \text{ sec}^{-1}\text{M}^{-1}$. The enzyme cleaved substrates having Arg-Arg, Pro-Arg, Ala-Arg, and Thr-Arg with increased K_m but with unchanged k_{cat} . In contrast, the enzyme displayed a dramatically lower k_{cat} for a Lys-Lys substrate with a smaller increase in K_m . Thus the two residues preceding the cleaved bond may play distinct roles in the selectivity of binding and cleavage of prohormone substrates.

The *Saccharomyces cerevisiae* Kex2 protein, a Ca^{2+} -dependent serine protease, is the only enzyme proven by genetic and biochemical evidence to cleave a prohormone (pro- α -factor) at pairs of basic residues in the eukaryotic secretory pathway (1-3). The high specificity of Kex2 protease, homologous to the subtilisin family (4, 5), contrasts with the broad specificity of previously known members of the family, all of which are degradative enzymes. Kex2 and its mammalian homologues furin (5, 6), PC2 (7, 8), and PC3 (also known as PC1; refs. 9 and 10) are more similar to each other than to the degradative subtilisins. The hypothetical roles of this "Kex2 subfamily" in processing secretory precursors in animal cells are under investigation in many laboratories. Properties of the partially purified yeast enzyme have served as a point of comparison with activities that cleave proinsulin (11) and proalbumin (12). Analysis of purified Kex2 protease should help define the nature and structural basis of substrate specificity in the Kex2 subfamily, the biological roles of Kex2 and its homologues, and the pathway of biosynthesis and posttranslational modification of these enzymes.

Purification of the native, transmembrane form of Kex2 protease was frustrated by lability of the C-terminal cytosolic tail to proteolysis (3). Because deleting the C-terminal 200 codons of *KEX2* resulted in appearance of enzyme at the cell surface (5), a study was undertaken to define the minimal

sequence that encodes active enzyme (ref. 13; P. Gluschkof and R.S.F., unpublished work). When strains expressing active, C-terminally truncated Kex2 were grown in buffered medium, activity accumulated in the medium in a soluble and stable form. Here we report purification and characterization of secreted, soluble Kex2 protease (ss-Kex2).

MATERIALS AND METHODS

Expression System. ss-Kex2 was purified from the culture media of strains CB023 and KRY77-3B carrying plasmid pG5-KEX2 Δ C3 (Fig. 1). Strain CB023 has genotype *MATa pep4::HIS3 prb1::hisG prc1 Δ ::hisG ura3 leu2 trp1 ade2 Gal⁺ cir⁰*. Strain KRY77-3B is isogenic except that it is *MATa PRB1 PRC1 ste13 Δ ::LEU2 cir⁺*. Strains, plasmids, and complete descriptions are available from the authors.

Media, Buffers, and Reagents. Each liter of 1040 medium (pH 7.2) contained 1.7 g of yeast nitrogen base without amino acids and $(\text{NH}_4)_2\text{SO}_4$ (Difco), 1.32 g of $(\text{NH}_4)_2\text{SO}_4$, 5 g of NH_4Cl , 5 g of vitamin assay Casamino acids (Difco), 8.37 g of [bis(2-hydroxyethyl)imino]tris(hydroxymethyl)methane (Bistris) base, 20 g of D-glucose, 0.12 g of L-tryptophan, and 0.24 g of Ade-HCl. Buffer Q was 40 mM Bistris-HCl, pH 7.0/10% (vol/vol) glycerol. Buffer SA was 20 mM Na-Mes, pH 5.5/0.1 mM Na-EDTA/10% glycerol. Buffer SB was SA with 500 mM NaCl and without EDTA. Buffer SC was SA lacking EDTA, adjusted to pH 5.3. Ac-Pro-Met-Tyr-Lys-Arg-methylcoumarin amide (-MCA) was a gift of J. Thorner (University of California, Berkeley). Other peptidyl-MCA substrates and 7-amino-4-methylcoumarin (AMC) were from Peptides International (Louisville, KY). Buffers and common reagents were from Calbiochem and Sigma.

Assay of Kex2 Activity. The standard assay contained, in 50 μl , 200 mM Bistris-HCl (pH 7.0), 1 mM CaCl_2 , 0.01% (vol/vol) Triton X-100, 0.5% (vol/vol) dimethyl sulfoxide, and 100 μM *tert*-butoxycarbonyl (Boc)-Gln-Arg-Arg-MCA. Reactions were initiated by adding enzyme (≤ 75 units), incubated at 37°C for 4 min, and terminated by placing tubes in ice/water and adding 0.95 ml of 0.125 M ZnSO_4 . Released AMC was determined with a Perkin-Elmer LS-5B fluorimeter (λ_{ex} 385 nm; λ_{em} 465 nm). One unit of Kex2 activity was defined as release of 1 pmol of AMC per min. Protein was determined with bicinchoninic acid (Pierce).

Analysis of Kinetic Data. Enzyme stability and steady-state kinetic assays were performed in 2.5 ml at 37°C in a magnetically stirred cuvette (1 cm^2) on a temperature-controlled ($\pm 0.1^\circ\text{C}$) fluorimeter stage. Emission was recorded by an IBM PS/2 computer. Data were analyzed with ENZFITTER 1.05 (Biosoft): enzyme stability with a first-order rate equation, pK_a determination with limits of 0 and 1, and steady-state parameters with Eadie-Hofstee plots.

Abbreviations: ss-Kex2, secreted, soluble Kex2 protease; MCA, methylcoumarin amide; AMC, 7-amino-4-methylcoumarin; DPAP, dipeptidyl aminopeptidase.

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RESULTS

Expression of ss-Kex2. When the transmembrane domain and C-terminal cytosolic tail of Kex2 were deleted, active truncated enzyme appeared at the cell surface but not in the culture medium (5). Recently, substantial C-terminally truncated Kex2 protease was found in the culture medium when buffered to pH ≥ 6 during fermentation (ref. 13; P. Gluschankof and R.S.F., unpublished work). Of several deletions tested, expression of *KEX2 Δ C3*, encoding the first 613 residues of Kex2 plus three additional residues (Fig. 1), led to the highest levels of thermostable secreted activity (data not shown). For purification, transcription of *KEX2 Δ C3* was driven by the *TDH3* promoter (3) on a multicopy plasmid in strain CB023, which had disruptions of the genes encoding vacuolar proteases A and B and carboxypeptidase Y (14). Important features of the medium were use of Bistris as a buffer and low sulfate to promote binding of the enzyme to Q Sepharose. Cell growth and accumulation of enzyme activity were improved by using 0.5% Casamino acids.

Purification of ss-Kex2. Due to the small amount of protein released by yeast cells into culture medium, the specific activity of ss-Kex2 in the medium (Table 1) was 100-fold higher than that of wild-type Kex2 protease in cell extracts when expressed using a similar vector (3). Therefore, purification of ss-Kex2 to near homogeneity required only a 10-fold enrichment. Enzyme was recovered rapidly by adding Q Sepharose resin directly to diluted medium. This afforded a 250-fold concentration of activity in <1 hr with nearly 100% yield (Table 1). Chromatography on S Sepharose removed contaminants not eliminated by anion exchange. Finally, HPLC on Mono Q yielded two closely eluted peaks of activity. By SDS/PAGE, the first peak (A) contained a single polypeptide (69 kDa), which densitometry indicated was $>99\%$ pure (Fig. 2, lane 1). The second peak (B) contained, in addition, a 64-kDa band. Immunoblotting demonstrated that both species were antigenically related to Kex2 (data not shown). Because of the sharp elution of a single species of higher specific activity in peak A, this material (fraction IV-A) was characterized further.

Structural Analysis. Residues 144–438 of the predicted Kex2 sequence are about 30% identical to mature subtilisin, with the greatest conservation near catalytic residues (4, 5). Signal peptide cleavage of preprosubtilisin is followed by

Table 1. Purification of ss-Kex2

Fraction	Protein, mg	Volume, ml	Activity, units $\times 10^{-7}$	Specific activity, (units/mg) $\times 10^{-6}$	Yield, %
I	36	2200	12	3.3	100
II	21	8.7	12	5.6	98
III	4.9	10.0	3.7	7.6	31
IV-A	0.52	2.0	1.7	33	14
IV-B	0.32	2.0	0.76	24	6.3

Strain CB023(pG5-KEX2 Δ C3) was grown at 30°C in 10-ml volumes in 18 \times 150 mm tubes rotated at maximum speed on a Rollordrum (New Brunswick Scientific) because these conditions were optimal for accumulation of stable activity in the medium of stationary cells. Harvest and subsequent steps were performed at 0–4°C. 1,10-Phenanthroline (Serva) was added to 100 μ M and cells were removed by centrifugation at 3000 $\times g$ for 16 min. Clarified medium (fraction I, 2.2 liters) was diluted with 3 vol of 40 mM Bistris base to reduce ionic strength and adjust the pH to 7. Fast-flow Q Sepharose (Pharmacia; 60-ml packed volume) was added to the diluted culture medium and stirred for 1 min. Resin was collected by vacuum filtration on a sintered glass funnel, poured into a column (25 \times 120 mm), washed with 60 ml of buffer Q, and eluted with a 60-ml linear gradient from 0 to 1 M NaCl in buffer Q. Fraction II was eluted with a midpoint at ≈ 150 mM NaCl and was pressure-dialyzed (N₂ gas at 350 kPa) against buffer SC in a stirred cell with a YM30 membrane (Amicon). Dialysis removed a yellow contaminant whose presence correlated with loss of activity in the next step. Dialyzed fraction II was centrifuged (10 min at 420,000 $\times g$) in a Beckman TL100 ultracentrifuge, then pumped at 1 ml/min onto a column (10 \times 100 mm) of fast-flow S Sepharose (Pharmacia) equilibrated with buffer SC using an IsoPure LC (Perkin-Elmer). The column was washed sequentially with 1 ml of buffer SA and a 2-ml linear gradient from SA to 90% SA/10% SB. Fraction III was eluted at 100 mM NaCl in a 12.5-ml linear gradient from 10% to 50% SB and was pressure-dialyzed against buffer Q. Dialyzed fraction III was pumped at 1 ml/min onto a Mono Q column (Pharmacia; 5 \times 50 mm). The column was washed with 2 ml of buffer Q plus 135 mM NaCl and eluted with a 13-ml linear gradient to buffer Q plus 200 mM NaCl. Fraction IV was eluted in two peaks totaling 20% of initial activity. Peak A was eluted with a sharp midpoint at 150 mM NaCl and peak B was eluted with a midpoint at 158 mM NaCl. Peak A was pooled and aliquots frozen in liquid N₂ were stored at -80°C without loss of activity for 8 months. The response of purified enzyme to inhibitors was qualitatively the same as published with partially purified enzyme (3).

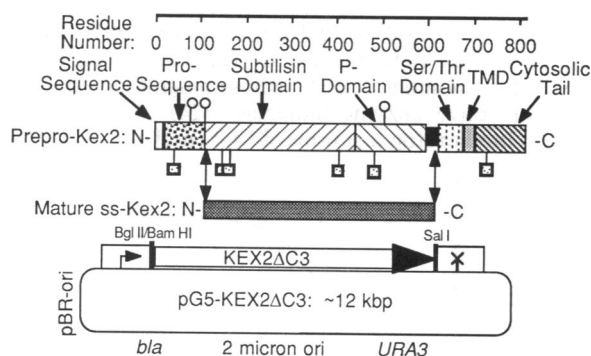


Fig. 1. Prepro-Kex2, mature ss-Kex2, and pG5-KEX2 Δ C3. Circles, Lys-Arg sites; squares, potential sites for Asn-linked glycosylation. P domain is described in ref. 13. TMD, transmembrane domain. pG5-KEX2 Δ C3 is the same as pAB23-KX22 (3) with the following exceptions. The 683-base-pair (bp) *Bgl* II–*Bam* HI fragment containing part of the polylinker and *lacZ* gene of M13mp18 was inserted into the *Bgl* II site of pAB23 to generate pG5. A *Sal* I adaptor encoding -Arg-Asn-Arg followed by tandem termination codons was inserted after codon 613 in *KEX2*, creating deletion Δ C3. The *KEX2 Δ C3* gene was cloned as a *Bam* HI–*Sal* I fragment into *Bgl* II/*Sal* I-cleaved pG5. The *TDH3* promoter and terminator of pG5-KEX2 Δ C3 are indicated by small arrow and "X".

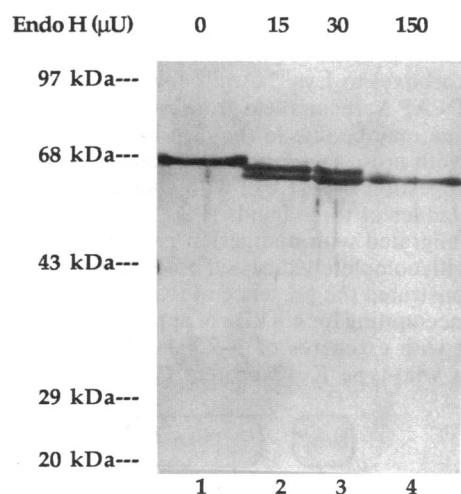


Fig. 2. Asn-linked carbohydrate in ss-Kex2. Samples (1 μ g) of fraction IV-A were heated at 97°C for 3 min in 2% (wt/vol) SDS/10 mM dithiothreitol and digested (37°C, 6 hr) with the indicated amounts (microunits, μ U) of endoglucosaminidase H (Endo H) (Boehringer Mannheim) in 1% (vol/vol) Triton X-100/0.2% SDS/100 mM sodium citrate, pH 5.8/10 mM dithiothreitol/10 mM Na-EDTA. Protein was precipitated, subjected to SDS/10% PAGE, and silver-stained.

Table 2. Edman degradation of ss-Kex2 from *STE13* wild-type and mutant strains

Strain	Phenylthiohydantoin amino acid derivative (yield, pmol)							
	1	2	3	4	5	6	7	8
<i>STE13</i>	Ala (102)	Pro (158)	Pro (52)	Pro (134)	Pro (54)	Pro (68)	Pro (72)	Met (50)
	Leu (89)		Ala (39)	Met (40)	Ala (49)	Met (40)	Asp (27)	Leu (38)
	Val (83)		Val (37)		Asp (26)	Ser (7)	Ser (7)	Ser (11)
<i>ste13Δ</i>	Leu (190)	Pro (130)	Val (213)	Pro (144)	Ala (204)	Pro (110)	Pro (143)	Met (203)

Enzyme (16 μ g) in water was analyzed in the presence of 2 mg of Polybrene with an Applied Biosystems model 475. Data (shown for cycles 1–8) were interpreted by A. Smith without knowledge of the predicted amino acid sequence.

autoproteolytic removal of the 77-residue pro domain (15). The predicted Kex2 sequence preserves this prepro organization, with potential autoproteolytic sites at Lys⁷⁹-Arg⁸⁰, Pro¹⁰²-Arg¹⁰³, and Lys¹⁰⁸-Arg¹⁰⁹. Dibasic and/or polybasic sites are conserved in the three mammalian homologues (6–10) at similar positions. Biosynthetic studies show that both wild-type Kex2 (16) and ss-Kex2 (P. Gluschankof and R.S.F., unpublished work) undergo rapid N-terminal proteolysis decreasing apparent mass by 9 kDa.

To identify the processing site in ss-Kex2, the N-terminal sequence of the purified enzyme was determined. Roughly equimolar peaks of phenylthiohydantoin (PTH) derivatives of Leu, Val, and Ala were released in the first round of Edman degradation (Table 2). The second round released only PTH Pro, whereas the third round produced similar amounts of three residues (PTH Val, Ala, and Pro). This suggested the presence of three N termini having Pro as the second residue. A search (QUEST 5.4, IntelliGenetics) using data from the first five cycles identified exactly three matches, corresponding to overlapping N termini beginning at residues 110, 112, and 114 (Fig. 3). These could be created by proteolysis at Lys¹⁰⁸-Arg¹⁰⁹, followed by partial removal of Leu¹¹⁰-Pro¹¹¹ and Val¹¹²-Pro¹¹³ dipeptides by a type IV DPAP, an enzyme that cleaves Xaa-Ala and Xaa-Pro dipeptides (17). Of two yeast type IV DPAPs, the Ste13 DPAP A, which removes Glu-Ala- and Asp-Ala- dipeptides after Kex2 cleavage of pro- α -factor (18), seemed more likely than vacuolar DPAP B (19) to encounter Kex2 protein. Edman degradation of ss-Kex2 purified from *ste13* null strain KRY77-3B containing pG5-KEX2 Δ C3 yielded a single, homogeneous sequence for at least 16 cycles (the first 8 are shown in Table 2). This corresponded precisely to the predicted sequence of Kex2 protein beginning at Leu¹¹⁰ (Fig. 3), proving that maturation of the N terminus of Kex2 requires a potentially autoproteolytic cleavage carboxyl to Lys¹⁰⁸-Arg¹⁰⁹ followed by trimming by the Ste13 DPAP A. Incomplete processing of ss-Kex2 from the *STE13* strain may be due to the high level of expression, as observed with pro- α -factor and pro- α -factor fusions (18, 20).

Partial digestion of ss-Kex2 with endoglucosaminidase H yielded a ladder of three bands (Fig. 2, lanes 2 and 3). The largest comigrated with undigested protein (lane 1) and the smallest with completely digested product (64.6 kDa, lane 4). This demonstrated the presence of two Asn-linked oligosaccharides, accounting for 4.4 kDa of apparent molecular mass, consistent with estimates of 3–5 kDa of Asn-linked carbohydrate in wild-type Kex2 protein (3, 5, 16). Because the

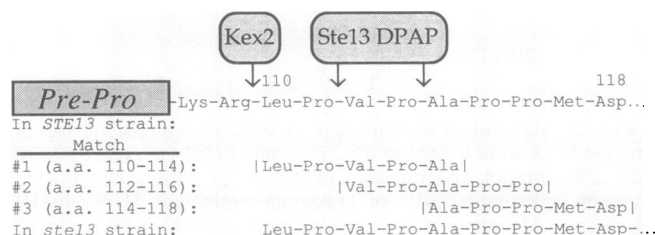


FIG. 3. N-terminal proteolytic maturation of Kex2 protein. DPAP, dipeptidyl aminopeptidase; a.a., amino acids.

mature portion of ss-Kex2 contains four potential sites for adding Asn-linked carbohydrate, two must be unmodified.

pH Dependence. Previous studies that used long endpoint assays to measure the pH dependence of Kex2 arrived at contradictory conclusions (21, 22). Profiles of pH vs. activity were bell-shaped and, in one study, a different pH optimum was found for each buffer used (22). Continuous monitoring of hydrolysis in several buffers at pH values from 5.0 to 9.5 showed that buffers had differential effects on the stability of Kex2 activity during incubation, with optimal stability for each buffer near its pK_a. For example, $t_{1/2}$ for loss of activity in Na-Hepes buffer at 37°C was 6 min at pH 7.0, 22 min at pH 7.5, and 5 min at pH 8.0. However, the enzyme was not intrinsically less stable at pH 7.0 or 8.0: $t_{1/2}$ for loss of activity in Bistris-HCl at pH 7.0 (standard assay) was 250 min.

To probe the pK_a of the chemical step and to avoid effects on binding interactions, cleavage of Boc-Gln-Arg-Arg-MCA, which does not titrate in the pH range examined, was assayed at a saturating concentration. Determining initial rates from 100-sec incubations rendered variations in stability in different buffers negligible. Under these conditions, Kex2 activity fit a simple sigmoidal pH titration curve (Fig. 4) like that of subtilisin (23) and other serine proteases (24). Activity increased sharply from pH 5 to pH 6.5, with half-maximal activity (pK_a) at pH 5.71 \pm 0.06, and was constant between pH 6.5 and pH 9.5. Activity as a function of pH deviated significantly from the theoretical curve only at pH 5, at which 14% of expected activity was observed. The drop in activity might be due to a change in the rate-determining step (24) or protonation of surface acidic residues, perhaps altering enzyme conformation or reducing Ca²⁺ affinity.

Active-Site Titration. Serine proteases follow a two-step mechanism. Acylation of the catalytic Ser accompanies release of the C-terminal fragment or reporter. This is followed by (usually) rate-limiting hydrolysis of the acyl enzyme. Thus, pre-steady-state release of the C-terminal leaving group creates an initial "burst" whose magnitude equals the number of active enzyme molecules (24). A quench-flow mixer was used to stop reactions of 10.0 pmol of ss-Kex2 with

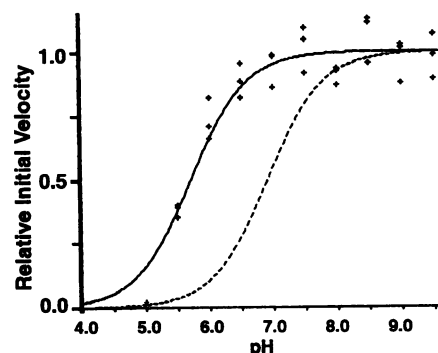


FIG. 4. pH profiles of ss-Kex2 and subtilisin. Assay mixtures contained 150 mM NaCl and 50 mM sodium salts of acetate (pH 5.0), Mes (pH 5.5, 6.0, and 6.5), Hepes (pH 7.0, 7.5, and 8.0), Bicine (pH 8.5 and 9.0), or Ches (pH 9.5). Assays (100 sec) were in triplicate. Dashed line, titration of subtilisin based on data in ref. 23.

a saturating concentration of Ac-Pro-Met-Tyr-Lys-Arg-MCA (20 μ M) at 4–500 msec after mixing (Fig. 5). At times prior to 12 msec, kinetics of AMC release were clearly pre-steady state, although the burst phase was nonlinear by 8 msec. Extrapolation of the linear phase (20–500 msec) revealed a 10.3 ± 0.1 pmol burst, indicating that fraction IV-A was fully active. Finding full activity in this preparation that was incompletely modified by the Ste13 DPAP demonstrated that neither removal nor retention of the Leu-Pro- and Val-Pro-peptides is required for Kex2 activity.

Substrate Specificity. Steady-state rate constants were measured for 10 peptidyl-MCA substrates (Table 3). Eight of nine substrates with Arg as the P₁ residue, including substrate 6 with Pro at P₂, had k_{cat} values nearly the same (70–180%) as that of substrate 1 (25 sec⁻¹), whose sequence is based on sites in pro- α -factor (3). The sole exception, substrate 9 (Boc-Gln-Gly-Arg-MCA), which differed from substrate 7 (Boc-Gln-Ala-Arg-MCA) by the absence of a methyl group, was cleaved with a 4-fold lower k_{cat} . Although k_{cat} was nearly constant for substrates 1–8, K_m values varied 360-fold. The lowest K_m values were for substrates 1 (2.2 μ M) and 2 (3.9 μ M), having Lys at P₂. Diarginyl tripeptide substrates 3 and 4 were cleaved with higher K_m (17 and 13 μ M). Although the K_m for substrate 6 (Boc-Val-Pro-Arg-MCA) was 10-fold higher than those for the diarginyl substrates, existence of a Pro-Arg cleavage site in pro-killer toxin suggests that cleavage at Pro-Arg may represent a physiologically relevant activity (26). Substrates 7, 9, and 8, with Ala, Gly, and Thr, respectively, at P₂, had the highest K_m values. Substitutions at P₃ had little effect. Comparable substrates with aromatic (Tyr), aliphatic (Leu), and strongly hydrophilic (Gln) residues at P₃ were cleaved with similar kinetics. Substrate 10, with Lys at P₁, had an \approx 100-fold lower k_{cat} than the first eight substrates, with Arg at P₁. The presence of Glu at P₃ in substrate 10 was unlikely to be responsible for this, given the insensitivity to P₃ substitutions. In addition, a Lys-Arg substrate with Asp at P₃ had a k_{cat} comparable to those of substrates 1–8 (unpublished data). Though the k_{cat}/K_m ratio of substrate 10 was the lowest, its K_m was only 14 times that of substrate 2.

The mammalian Kex2 homologue furin may be specific for cleavage at Arg-Xaa-(Lys/Arg)-Arg \downarrow (27). A comparison of known or inferred Kex2 cleavage sites suggests a preference for an aliphatic residue at P₄ (data not shown). Only substrate 1 has a principally aliphatic residue at P₄ (Met), although eight of the remaining nine are tripeptides having an N-terminal *t*-butyl, which might interact with a hydrophobic P₄ binding site much like a P₄ side chain. However, ss-Kex2 cleaved substrate 5, with Arg at P₄, with about the same kinetics as other Arg-Arg substrates. The P₄ side chain might neverthe-

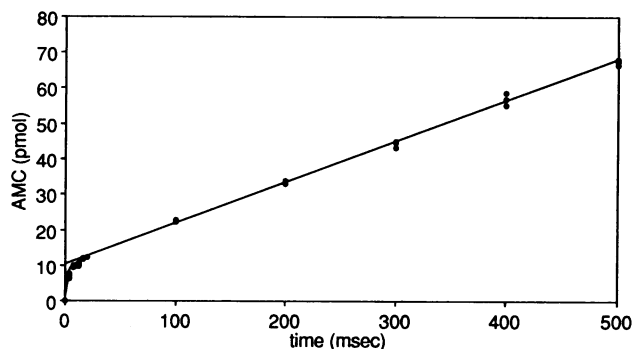


FIG. 5. Initial burst titration. Reactions were performed in triplicate for time points of 4–500 msec with a quench-flow apparatus (KinTek RQF-3). Protein concentration determined by amino acid analysis (4.67 μ M = 258 μ g/ml) agreed with bicinchoninic acid (256 μ g/ml) and Folin-phenol (264 μ g/ml) assays. Ten picomoles (based on amino acid analysis) of ss-Kex2 from strain CB023 in 37 μ l of standard reaction mixture without substrate was mixed with an equal volume of standard reaction mixture with 40 μ M Ac-Pro-Met-Tyr-Lys-Arg-MCA and quenched with 750 mM citric acid (pH 2.95). Detection of the leaving group by fluorescence made the assay extremely economical, using only 18 μ g of enzyme for 33 time points.

less be important in cleavage of native precursors in which the conformation of the P₄ side chain may be restricted.

DISCUSSION

Although studies of partially purified Kex2 have yielded useful information (1, 3, 21, 22), characterization of highly purified ss-Kex2 permits definitive analysis of the activity and structure of this enzyme. Indeed, conflicting claims about pH dependence and cleavage of Pro-Arg substrates (21, 22) and uncertainties in estimates of K_m values for Lys-Arg and Arg-Arg substrates (3) have now been resolved.

ss-Kex2 had higher activity in acidic conditions than most serine proteases, which exhibit half-maximal activity near pH 7. The pK_a values for the catalytic steps of subtilisin (6.9) and chymotrypsin (6.8) represent the ionization of the active-site histidine (23, 24). Single mutations in subtilisin that changed surface charges from negative to positive reduced the pK_a of k_{cat}/K_m by as much as 0.6 unit and were additive (23). Analogous features of Kex2 may be responsible for its reduced pK_a. Although the pH of the compartment in which Kex2 protease functions in yeast is unknown, these results suggest that the enzyme should be active in acidic conditions thought to prevail in secretory granules where prohormone processing occurs in mammalian cells (pH 5.7; ref. 28).

Table 3. Steady-state kinetic parameters for cleavage of peptidyl-MCA substrates

	Substrate						k_{cat} , sec ⁻¹	K_m , μ M	k_{cat}/K_m , sec ⁻¹ ·M ⁻¹	$\Delta\Delta G$, kcal·mol ⁻¹
	P ₅	P ₄	P ₃	P ₂	P ₁	P ₁ '				
1	Ac	Pro	Met	Tyr	Lys	Arg	25	2.2	11,000,000	0.00
2		Boc	Leu	Lys	Arg	MCA	23	3.9	5,900,000	0.40
3		Boc	Leu	Arg	Arg	MCA	45	17	2,600,000	0.90
4		Boc	Gln	Arg	Arg	MCA	21	13	1,600,000	1.2
5		Boc	Arg	Val	Arg	Arg	21	19	1,100,000	1.4
6		Boc	Val	Pro	Arg	MCA	31	150	210,000	2.5
7		Boc	Gln	Ala	Arg	MCA	26	210	120,000	2.8
8		Boc	Leu	Thr	Arg	MCA	18	800	22,000	3.8
9		Boc	Gln	Gly	Arg	MCA	6.1	320	19,000	3.9
10		Boc	Glu	Lys	Lys	MCA	0.19	55	3,500	5.0

Reactions, performed in duplicate or triplicate at seven substrate concentrations over a 64-fold range centered near K_m , were initiated by 100 fmol of enzyme and recorded for 100–500 sec. k_{cat} was based upon active-site titration (Fig. 5). Standard errors of k_{cat} and K_m averaged 8% and 20%, respectively. Classification of substrate residues as P₁', P₁, P₂, etc. was described in chapter 1 of ref. 24. Relative transition-state binding energies were calculated according to ref. 25: $\Delta\Delta G = -RT \ln (k_{\text{cat}}/K_m \text{ substrate } 1)/(K_m \text{ substrate } x)$. $\Delta\Delta G$ for any two substrates may be obtained by subtraction of the listed values.

Alterations at P₁ and P₂ appear to have different effects on k_{cat} and K_m , consistent with independent subsites for these residues, an expected feature if substrates bind in extended β -conformation as found with subtilisin (29) and proteinase K (30). The large effect on k_{cat} observed with the "conservative" substitution of Lys for Arg at P₁ in substrate 10 suggests that the P₁ binding site is highly specific for Arg. Release of AMC, not enzyme deacylation, is rate-limiting in the hydrolysis of this substrate (unpublished data), consistent with a reduced rate of formation of the acyl-enzyme due to nonproductive binding. The conformation required for the shorter Lys side chain to interact with hypothetical negatively charged groups in the P₁ binding pocket may position the carbonyl poorly for nucleophilic attack.

With Arg at P₁, substitutions at P₂ had little effect on the overall catalytic rate and thus are unlikely to affect the positioning of the reactive carbonyl. Pro at P₂, a residue that cannot fit into a strict β -conformation, resulted only in an effect on K_m . The single exception, Gly at P₂, reduced k_{cat} by a factor of 4, perhaps due to increased conformational flexibility in the substrate. Although the requirements for an efficient k_{cat} appear to be limited to the presence of Arg at P₁ and a residue other than Gly at P₂, pairwise comparisons between substrates that differ by one residue indicate that the nature of the P₂ residue is a major determinant of K_m and that residues beyond P₂ are less important. That nearly the same elevated K_m values were observed with Gly and Ala at P₂ suggests that discrimination of P₂ side chains depends upon more extensive contacts such as those contributed by Lys or Arg side chains. Comparison of substrate 9 with substrates 4 and 7 allows calculation of transition-state binding energies for specific groups in the P₂ side chain (25). Comparison of substrates 9 and 7 shows that loss of the methyl side chain costs 1.1 kcal/mol due to reduced k_{cat} . Loss of the rest of the Arg side chain costs only an additional 1.6 kcal/mol. These observations, and the fact that the P₂ binding pocket accommodates both Lys and Arg with only a small change in K_m , suggest that the primary determinants for discrimination at P₂ may be limited to charge neutralization and steric exclusion, possibly of β -branched side chains. Thr at P₂ resulted in a 4-fold increase in K_m relative to Ala at P₂, and in fact, substrate 6 had the highest K_m .

Discrimination by a processing enzyme ultimately depends on its specificity for cleaving desired vs. undesired bonds. Relative transition-state binding energies ($\Delta\Delta G$, Table 3) measure the selectivity of catalysis (25). The high degree of selectivity of ss-Kex2 is illustrated by $\Delta\Delta G$ values up to 3 kcal/mol for single amino acid substitutions at P₂ and by a $\Delta\Delta G$ of 4–5 kcal/mol for discrimination between Lys-Arg and Lys-Lys substrates. The high specificity constants (k_{cat}/K_m) of ss-Kex2 with its best known substrates can be compared with those of other proteases. The best synthetic amide substrates for subtilisin (23), trypsin (31), and factor Xa (32) are cleaved with 2, 6, and 8 times the k_{cat} , and 80, 20 and 50 times the K_m , of the best synthetic substrate of ss-Kex2. Thus Kex2 has achieved catalytic specificity by reducing K_m with only a modest decrease in turnover number.

Kinetic characterization of ss-Kex2 with peptidyl-MCA substrates, while informative, probes only one aspect of specificity. *In vivo*, the enzyme is concentrated 30-fold relative to other secretory proteins (16) in a late Golgi compartment (33, 34) where it might function as part of an assembly of processing enzymes. Native substrates may be cleaved with different kinetics than corresponding synthetic substrates due to substrate secondary structure or interactions distant from or C-terminal to the cleavage site. Further analysis of ss-Kex2 with native and synthetic substrates and

inhibitors should help to elucidate the important elements of substrate recognition in prohormone processing.

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- Julius, D., Brake, A., Blair, L., Kunisawa, R. & Thorner, J. (1984) *Cell* **37**, 1075–1089.
- Fuller, R. S., Sterne, R. E. & Thorner, J. (1988) *Annu. Rev. Physiol.* **50**, 345–362.
- Fuller, R. S., Brake, A. & Thorner, J. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 1434–1438.
- Mizuno, K., Nakamura, T., Ohshima, T., Tanaka, S. & Matsuo, H. (1988) *Biochem. Biophys. Res. Commun.* **156**, 246–254.
- Fuller, R. S., Brake, A. J. & Thorner, J. (1989) *Science* **246**, 482–486.
- Van den Ouweland, A. M. W., van Duijnhoven, H. L. P., Keizer, G. D., Dorssers, L. C. J. & Van de Ven, W. J. M. (1990) *Nucleic Acids Res.* **18**, 664.
- Smeekens, S. P. & Steiner, D. F. (1990) *J. Biol. Chem.* **265**, 2997–3000.
- Seidah, N. G., Gaspar, L., Mion, P., Marcinkiewicz, M., Mbikay, M. & Chrétien, M. (1990) *DNA Cell Biol.* **9**, 415–424.
- Smeekens, S. P., Avruch, A. S., LaMendola, J., Chan, S. J. & Steiner, D. F. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 340–344.
- Seidah, N. G., Marcinkiewicz, M., Benjannet, S., Gaspar, L., Beaubien, G., Mattei, M. G., Lazure, C., Mbikay, M. & Chrétien, M. (1991) *Mol. Endocrinol.* **5**, 111–122.
- Davidson, H. W., Peshavaria, M. & Hutton, J. C. (1987) *Biochem. J.* **246**, 279–286.
- Brennan, S. O. & Peach, R. J. (1988) *FEBS Lett.* **229**, 167–170.
- Fuller, R. S., Brenner, C., Gluschankof, P. & Wilcox, C. A. (1991) in *Methods in Protein Sequence Analysis*, eds. Jörnvall, H., Höög, J.-O. & Gustavsson, A.-M. (Birkhäuser, Basel), pp. 205–214.
- Jones, E. W. (1991) *J. Biol. Chem.* **266**, 7963–7966.
- Power, S. D., Adams, R. A. & Wells, J. A. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 3096–3100.
- Wilcox, C. A. & Fuller, R. S. (1991) *J. Cell Biol.* **115**, 297–307.
- Kreil, G. (1990) *Trends Biochem. Sci.* **15**, 23–25.
- Julius, D., Blair, L., Brake, A., Sprague, G. & Thorner, J. (1983) *Cell* **32**, 839–852.
- Roberts, C. J., Pohl, G., Rothman, J. H. & Stevens, T. H. (1989) *J. Cell Biol.* **108**, 1363–1373.
- Brake, A. J. (1989) in *Yeast Genetic Engineering*, eds. Barr, P. J., Brake, A. J. & Valenzuela, P. (Butterworth, Boston), pp. 269–280.
- Achstetter, T. & Wolf, D. H. (1985) *EMBO J.* **4**, 173–177.
- Mizuno, K., Nakamura, T., Ohshima, T., Tanaka, S. & Matsuo, H. (1989) *Biochem. Biophys. Res. Commun.* **159**, 305–311.
- Russell, A. J. & Fersht, A. R. (1987) *Nature (London)* **328**, 496–500.
- Fersht, A. (1984) *Enzyme Structure and Mechanism* (Freeman, New York), 2nd Ed.
- Wilkinson, A. J., Fersht, A. R., Blow, D. M. & Winter, G. (1983) *Biochemistry* **22**, 3581–3586.
- Bostian, K., Elliot, Q., Bussey, H., Burn, V., Smith, A. & Tipper, D. J. (1984) *Cell* **36**, 741–751.
- Bresnahan, P. A., Leduc, R., Thomas, L., Thorner, J., Gibson, H. L., Brake, A. J., Barr, P. J. & Thomas, G. (1990) *J. Cell Biol.* **111**, 2851–2859.
- Anderson, R. G. W. & Orci, L. (1988) *J. Cell Biol.* **106**, 539–543.
- Kraut, J. (1977) *Annu. Rev. Biochem.* **46**, 331–358.
- Betzel, C., Pal, G. P. & Saenger, W. (1988) *Eur. J. Biochem.* **178**, 155–171.
- Gráf, L., Hegyi, G., Likó, I., Hepp, J., Medzihradsky, K., Craik, C. S. & Rutter, W. J. (1988) *Int. J. Peptide Protein Res.* **32**, 512–518.
- Lottenberg, R., Hall, J. A., Pautler, E., Zupan, A., Christensen, U. & Jackson, C. M. (1986) *Biochim. Biophys. Acta* **874**, 326–336.
- Franzusoff, A., Redding, K., Crosby, J., Fuller, R. S. & Schekman, R. (1991) *J. Cell Biol.* **112**, 27–37.
- Redding, K., Holcomb, C. & Fuller, R. S. (1991) *J. Cell Biol.* **113**, 527–538.